ONTARIO MINISTRY OF THE ENVIRONMENT LABORATORY SEDIMENT BIOLOGICAL TESTING PROTOCOL

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Mention of trade names in this document does not constitute endorsement by Environment Ontario.

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FOREWORD

The protocol provided in this document is to replace the previous Provincial Sediment Bioassessment Protocol (Lomas and Krantzberg, 1988). The procedures can be used in the evaluation of contaminated sediments by providing information on lethal and sublethal biological effects. Test applications include the assessment of sediments at remedial action plan sites, general surveillance and monitoring and spills action. In addition, it is intended to be used for the assessment of acute toxicity as prescribed in the "Guidelines for the Protection and Management of Aquatic Sediment Quality in Ontario" (Persaud *et al.*, 1992).

This document provides a background and rationale for sediment bioassessment and provides detailed methodology for conducting sediment bioassays and for the rearing and maintenance of test organisms.

SECTION 1: BACKGROUND

In Ontario, contaminated sediment has long been identified as a major environmental concern (IJC, 1985). In 1976, sediment evaluation procedures were first introduced with the Open Water Disposal Guidelines (Persaud and Wilkins, 1976). In 1983, the Ontario Ministry of the Environment (OMOE) embarked on the In-Place Pollutants Program to develop broad evaluation techniques for sediment (Lomas and Persaud, 1987). This led to the development of comprehensive sediment quality guidelines and the development of laboratory sediment bioassay procedures to assist in sediment evaluation.

In 1988, the OMOE developed a sediment bioassessment protocol (Lomas and Krantzberg, 1988) to measure acute toxic effects of sediments (namely mortality) on juvenile fathead minnows and mayfly nymphs. Further research and development was conducted to address longer-term and more subtle effects of contaminated sediments. This research also addressed the effects of various test procedures on the outcome of the bioassay by examining factors such as test duration, organism density, diet, age and size of the organisms, and sediment physical characteristics.

The sediment bioassessment procedures outlined in this document are capable of providing information for a variety of sediment applications including general monitoring and surveillance and sediment evaluation for contaminated sites, "Areas of Concern" and spill sites (Figure 1). The bioassays include short term, Acute Lethality Tests (see Section 6.1) as specified in Persaud *et al.* (1992) and longer-term bioassays to assess sublethal and bioaccumulative effects.

SECTION 2: ESTABLISHING THE NEED FOR SEDIMENT BIOASSAYS

Laboratory bioassays can be used to assess the significance of contaminated sediments on aquatic biota by measuring biological effects. The laboratory procedures described in this document can be used to measure the degree of toxicity associated with contaminants in sediment, the area affected and the possible nature of the problem.

Laboratory bioassays are a complementary tool to support field data and as such should be carried out where field data suggest that sediment quality has been degraded. The degree of degradation can be identified using a battery of biological tests that examine both lethal and sublethal effects and chemical bioaccumulation. This will provide an overall evaluation of the area in question.

In addition the Guidelines for the Protection and Management of Aquatic Sediment Quality in Ontario (Persaud *et al.*, 1992) recommend acute bioassays be carried out in the specific circumstance where sediment concentrations exceed the Severe Effect Level guidelines, for example at "hot spots" (Persaud *et al.*, 1992). The SEL is the sediment concentration of a compound that would be detrimental to the majority of benthic species. The bioassays examine lethality of mayflies and minnows over a 10-day exposure period.

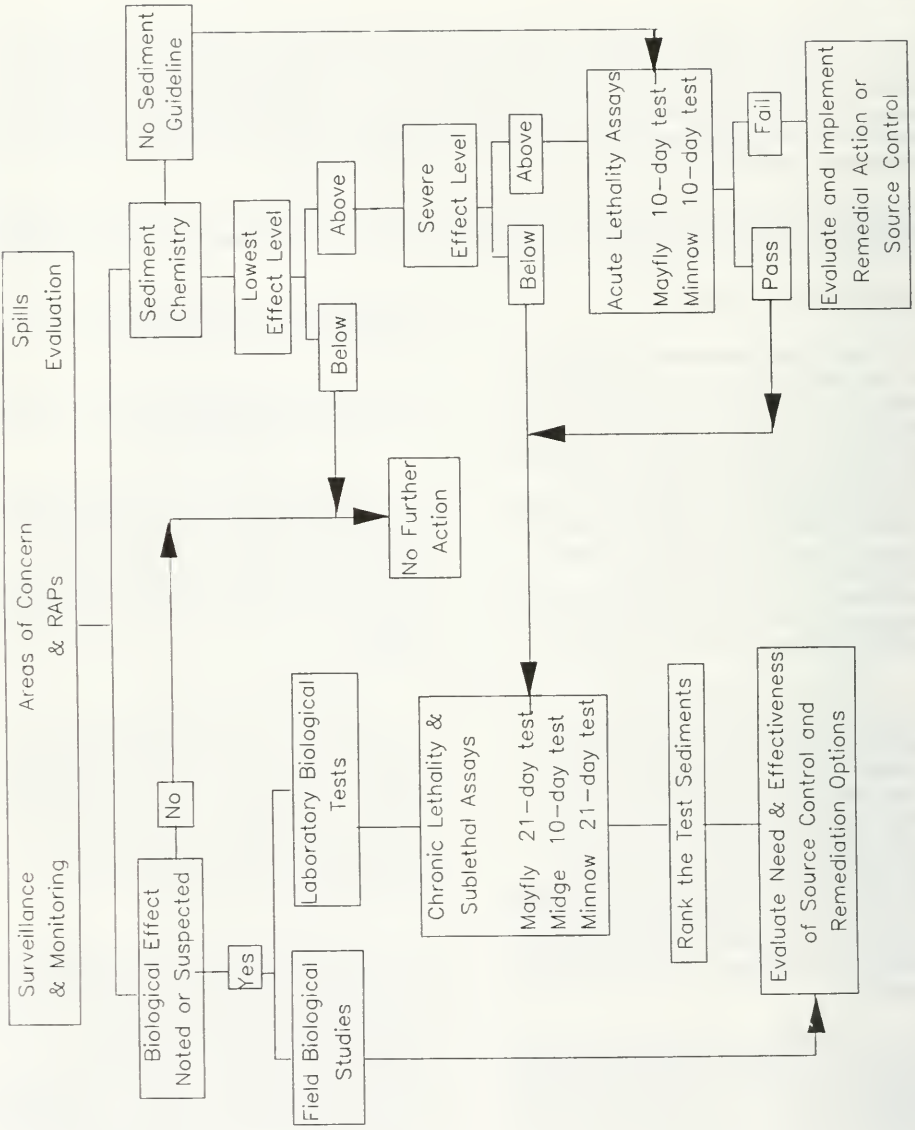

Figure 1 Flow chart outlining the applications of sediment bioassays.

For a more comprehensive examination of sediment toxicity such as for general monitoring and surveillance or where the chemical concentrations exceed the Lowest Effect Level guidelines (the sediment chemical concentration that could be tolerated by the majority of benthic organisms), longer-term laboratory tests may be recommended to examine lethal and sublethal effects, and chemical bioaccumulation. Such tests are described in this document.

Sediment management decisions are in part based upon the outcome of the laboratory sediment bioassays. Sediments that are lethal to any of the test species would be considered a candidate for immediate management action. Sediments that are not lethal but exhibit significant growth impairment are recommended for further assessment such as field verification studies or additional laboratory tests before an appropriate course of action is selected.

Sediment bioassay tests can be used for the following purposes:

(1) To assess the effects of sediment contamination by measuring biological effects (lethal and sublethal), and to assess the spatial and temporal trends within a given area by ranking the test results.

(2) To assess the potential for uptake and food chain transfer of sediment-bound contaminants by measuring uptake under laboratory conditions.

(3) To assess the effectiveness of remedial measures.

SECTION 3: RATIONALE FOR SEDIMENT BIOASSESSMENT METHODOLOGY

3.1 TEST METHODOLOGY

The sediment bioassay methodology has been selected on the basis of simplicity and reproducibility in order to facilitate their use by other laboratories. Factors that could affect the outcome of the bioassay such as size of the test chambers, density of the test organisms, test duration, size and age of the test organisms and diet during the test were investigated to determine optimum design (Bedard, 1989; Krantzberg, 1990).

The bioassays are static, single-species tests using whole-sediment. Control and test sediments are placed into glass jars and dechlorinated water is added to achieve a 4:1 (v:v), water to sediment ratio (Figure 2). Cultured or reared test organisms are randomly added to the test chambers and the tests are run with a minimum of three replicates. The tests are carried out in a 20°C water-bath and a 16:8 hour, light:dark photoperiod. Table 1 provides a general description of the battery of tests.

Whole-sediment bioassays examine all possible routes of chemical exposure i.e. overlying water, interstitial water and sediment particles. This type of test requires the least amount of manipulation prior to testing relative to tests using sediment extracts, elutriates or interstitial water. Both laboratory and field studies rely on physical and chemical properties on bulk sediment and are used to examine biological effects on the benthos, thus allowing for more direct comparisons. The latter is also the premise for the development of the Provincial Sediment Quality Guidelines (Persaud *et al.*, 1992).

The tests use organisms from different trophic levels, of different ecological needs and relative sensitivities, and measure multiple endpoints in order to assess site-specific sediment-related concerns as recommended by IJC (1988), Giesy *et al.* (1988) and Munawar *et al.* (1989).

Other types of exposures have also been described in the literature including interstitial water and elutriate tests for assessing sediment quality (Malueg *et al.*, 1984; Giesy *et al.*, 1988). These tests are commonly used to screen a large number of samples and are rapid, acute tests using surrogate species e.g. *Daphnia* (Giesy and Hoke, 1989; 1990). Elutriate tests may have a more direct application and have been used to assess the potential impact associated with dredging activities and the damage that may arise through the resuspension of bottom sediment (USEPA/USACOE, 1977). The utility of pore

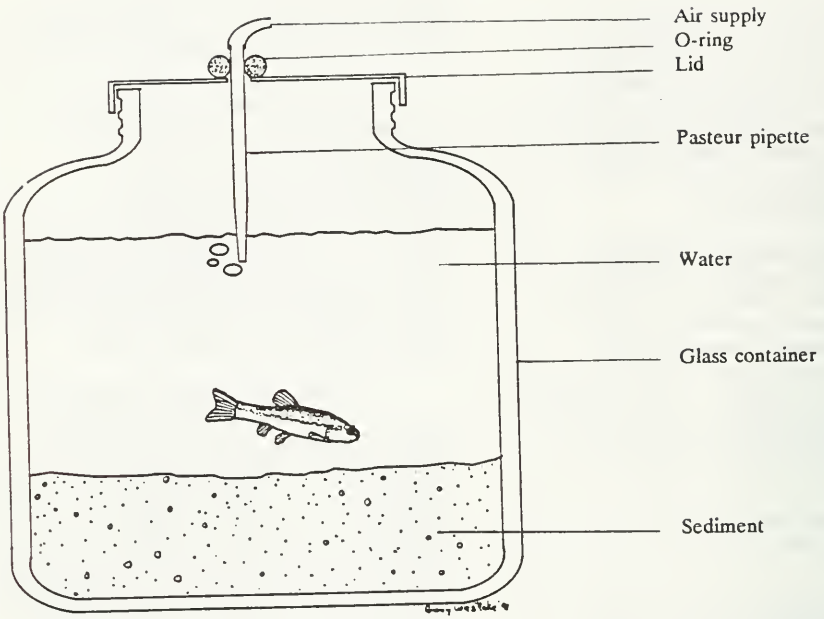

Figure 2 Schematic diagram of the test chamber.

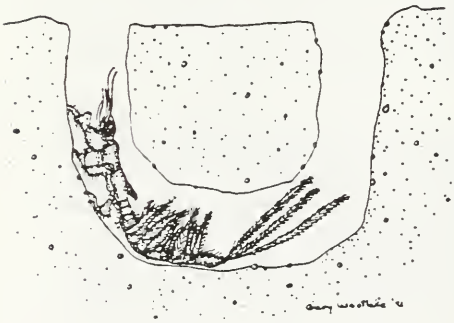

Figure 3 Line drawing of the burrowing mayfly, *Hexagenia* sp.

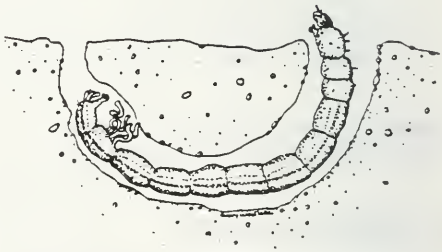

Figure 4 Line drawing of the midge larvae, *Chironomus* sp.

Table 1: Test Parameters for the Battery of Biological Tests

Test Species	Ecological Niche	Test Duration	Endpoint	# of Organisms Per Replicate	Feeding
Hexagenia limbata (mayfly)	benthic deposit & suspension feeder	10 day	acute mortality	10	no
		21 day	chronic mortality growth		
Chironomus tentans (midge)	benthic deposit & suspension feeder	10 day	chronic mortality growth	15	yes
Pimephales promelas (minnow)	water-column bottom forager	10 day	acute mortality	10	yes
		21 day	chronic mortality bioaccumulation		

water or elutriate tests for determining the toxicity of sediment remains to be fully examined and methodology for removing interstitial water has not been standardized (Hoke and Prater, 1980; Chapman et al., 1987).

3.2 SELECTION OF TEST ORGANISMS

The test organisms have been selected on the basis of their use by other laboratories and factors such as their sensitivity to contaminants, availability, ecological importance, life-history characteristics and habitat tolerance. Presently, the OMOE performs sediment tests using mayflynymphs, chironomid larvae and juvenile fathead minnows that are laboratory cultured or reared. In the future, other test species may be added or substituted, based on continuing research and development.

Background information supporting the selection of the test species is summarized below.

MAYFLY NYMPH (*Hexagenia limbata*)

The OMOE is conducting a sediment bioassay using early instar mayfly nymphs which are laboratory-reared from field-collected eggs using techniques similar to those described by Friesen (1981). Previous procedures (Lomas and Krantzberg, 1988) have been revised to include a measurement of mayfly growth for a period of 21 days.

Sediment bioassays using mayfly nymphs have examined mortality, growth, moulting frequency and preference/avoidance behaviour in whole-sediment tests (Nebeker et *al.*, 1984; Friesen *et al.*, 1983; Prater and Anderson, 1977; Malueg *et al.*, 1983, 1984).

The life cycle of burrowing mayflies (Ephemeroptera: Ephemeridae) include egg, nymph, sub-imago and imago or winged adult (Needham *et al.*, 1935). Since *Hexagenia* (Walsh) are benthic and burrow and feed directly from the substrate, they are a valuable test organism for assessing sediment quality.

Mayfly nymphs are commonly associated with soft, fine-textured and organically rich sediments which allow for adequate burrow formation (Wright and Mattice, 1981) (Figure 3). Smaller nymphs have a greater tolerance to a broader range of sediment types (Hunt, 1953). Bedard (1989) exposed reared, early instar ;dl;sl

nymphs to a number of clean sediments with a range of grain size and organic content and found the nymphs were capable of surviving and growing in sediment with a 91% sand content and TOC of 5.4 mgg^{-1} but tend to be limited by a high sand content (>80%) when combined with very low TOC (<2.0 mgg^{-1}) (D. Bedard, unpublished data). Normally these sediments do not support mayfly populations and would not be appropriate for testing with the mayfly nymphs e.g. beach sand, inert tailings.

The nymphs are deposit-feeders that ingest detritus, organic matter and sediment particles (Zimmerman and Wissing, 1978). Potential routes of exposure to sediment-bound contaminants include the sediment, interstitial water and overlying water (Giesy and Hoke, 1989, Landrum and Poore, 1988).

Hexagenia sp. has been recommended by the IJC (1989) as the benthic indicator organism representative of mesotrophic conditions primarily as a result of the nymph's sensitivity to oxygen depletion resulting from organic enrichment (Reynoldson *et al.*, 1989).

MIDGE LARVAE (*Chironomus tentans*)

The OMOE partial life cycle bioassay using cultured *Chironomus tentans* larvae is based on published procedures (Giesy *et al.*, 1988; Nebeker *et al.*, 1984) and experiments conducted in our laboratory which indicated that feeding is a necessary requirement of the test (Bedard, 1989).

Partial life cycle sediment bioassays examining larval survival and growth have been conducted using 10 - 12 day old, second instar larvae for a period of 10 to 14 days (Nebeker *et al.*, 1984; Adams, 1987; Giesy *et al.*, 1988). Larvae in the second instar are more sensitive than later instars (3rd and 4rth instar) (Gauss *et al.*, 1985). Whole-life cycle tests that measure adult emergence and fecundity are less reliable (Giesy and Hoke, 1989).

The benthic invertebrate *Chironomus tentans* (Diptera: Chironomidae) or fresh-water midge lives in a tunnel formed within surficial sediment while in the larval stage (Adams and Heidolph, 1985) (Figure 4). It completes its life cycle within 30 days at 20°C under laboratory conditions and mass cultures are easily maintained (Townsend *et al.*, 1981; ASTM, 1990).

Since larvae live in close proximity to the sediment they are exposed to contaminants in the interstitial and overlying waters (Adams, 1987). Larvae graze on detritus and filter food particles from the overlying water. Studies indicate that *Chironomus tentans* is capable of inhabiting sediment with a range of physical compositions and enhanced growth is common for coarser substrates with a >80% sand fraction (Bedard, 1989).

JUVENILE FATHEAD MINNOW (*Pimephales promelas*)

In the OMOE revised protocol a 21-day static sediment bioassay is used for determining mortality and availability of sediment-associated contaminants, in addition to a 10-day acute lethality test. Similar sediment bioassays have been described elsewhere e.g. Mac *et al.* (1990). Fathead minnows have been commonly used in aqueous exposures both acute (ASTM, 1988) and chronic (ASTM, 1988). Standard mass culturing techniques are available for fathead minnows (USEPA, 1978).

Lomas and Krantzberg (1988) developed a sediment bioassay methodology that examined the effects of contaminated sediments on juvenile fathead minnows (*Pimephales promelas*). The tests measured short-term mortality and chemical bioaccumulation. The potential sources of contaminants to fathead minnows are the bottom sediment, suspended sediment and the overlying water. Fathead minnows actively forage at the sediment-water interface. This behaviour can suspend sediments in the water column which may increase exposure to contaminants. Accumulation could occur through ingestion, adsorption and absorption.

3.3 SELECTION OF BIOLOGICAL ENDPOINTS

Laboratory sediment bioassays provide information which can be used with physical and chemical data to provide the basis of a comprehensive sediment evaluation. The OMOE sediment bioassay protocol is designed to provide information on the lethal and sublethal effects of the sediment and biological availability of contaminants. These are measured as mortality, growth impairment and tissue contaminant concentrations. The types of endpoints used in the protocol are discussed below.

LETHALITY

Lethality can be measured as acute (short-term) or chronic (long-term). Mortality assessments vary only in the method of differentiating between live and dead animals. Mortality is compared among the test sediments and the control(s).

Since mortality can be the result of physical as well as chemical conditions, mortality data cannot be interpreted independent of physical data.

SUBLETHAL GROWTH EFFECTS

Sublethal effects were not considered in the previous sediment bioassay protocol (Lomas and Krantzberg, 1988). The method was capable only of identifying sediments that were acutely toxic where there were few or no organisms living. Jaagumagi and Persaud (1991) concluded that, in most cases, mortality in the sediment bioassay test organisms occurred only for those sediments where nothing survived in the field and that a cursory examination of the sediments for the presence of benthic organisms would provide the same information. The sediment bioassay could not distinguish between sediments with a healthy diverse benthic community and those where only the most pollutant-tolerant species could survive.

In order to identify sediments with moderate contamination that are incapable of supporting diverse benthic communities, longer-duration or partial life-cycle biological tests that are capable of identifying sublethal effects are required. There are many sublethal responses such as those affecting organism growth, development, behaviour or reproduction. However, growth is the most immediate and easily measured response and this endpoint has been incorporated into the protocol.

CHEMICAL BIOAVAILABILITY

Since tissue analysis of exposed organisms provides information on the relative chemical availability among different test sediments, it has been incorporated into the protocol. The bioaccumulation potential of sediment-associated contaminants will vary with chemical availability, species physiology, partitioning behaviour of the chemical and the ability of the organism to metabolize the compound.

Chemical concentrations in the animal and sediment are compared and bioaccumulation factors (BAFs) are calculated. However it must be pointed out that, without further research, tissue concentrations cannot be related with any degree of confidence to any measured lethal or sublethal effect. Nor is there sufficient information at this time to relate tissue levels in test organisms to those that would be found in biota collected in the field.

SECTION 4: SELECTION OF CONTROL SEDIMENTS

The use of control sediments provides a measure of quality assurance both for the experimental design and for the health of the test organisms. The measurement of biological responses to standard controls allows for intercomparisons among different tests and among different laboratories. It is preferable to have three types of control sediments. These include a clean or negative control, a site reference control and a positive or toxic control.

4.1 NEGATIVE CONTROL

The clean or negative control sediment is used in all laboratory sediment bioassays and remains constant from test to test. It is collected from the field in a relatively contaminant-free area and must be physically and nutritionally suitable for normal growth and survival of the test organisms. The sediment is used to evaluate the

suitability of the test conditions and methodology, to the test organisms and therefore should provide a "benchmark" for comparing the response in other sediments. Negative control mortality must not exceed 15% for mayflies and fathead minnows and 25% for chironomids.

4.2 REFERENCE CONTROL

The reference control sediment is obtained from the study area but from a site that is removed from the source of pollution being investigated. Normally the reference sediment is collected in a clean upstream location and is indicative of the natural background levels for that locality. The reference control sediment should be comparable in physical attributes to the test sediments in order to differentiate toxic or sublethal effects resulting from exposure to above ambient contaminant levels from the effects of the natural physical characteristics of the test sediment.

4.3 POSITIVE CONTROL

A positive or reference toxicant test has a known toxic response and can be used as a quality control measure to assess organism health and sensitivity and test accuracy over time. Aqueous reference toxicity tests have been well-defined for effluent toxicity tests and measure acute lethality of a test species to chosen chemical concentrations (Environment Canada, 1990). Similar test procedures can be routinely applied to benthic species with appropriate modifications e.g. artificial substrates (Henry *et al.*, 1986). The OMOE has examined the use of cadmium and copper in aqueous exposures (D. Bedard, unpublished data).

SECTION 5: DATA INTERPRETATION

The data are analyzed statistically in order to identify differences in mortality and growth. Comparisons are made among the test sediments and the control(s) using One-way analysis of variance (ANOVA) and comparative tests such as Tukey's multiple range *t*-test or planned comparisons and Dunnett's *t*-test for comparisons between the control and each test sediment. Analysis is made on appropriately transformed data. Sediments that cause high mortality (> 60%) are excluded from the statistical analysis of growth effects due to possible biases. The initial starting weight of the test species is taken into consideration in the overall analysis. Coefficients of variation (C.V.%) are calculated for each endpoint as a measure of test precision. Spearman rank correlation analysis is used to investigate the correlation among the different endpoints for each species and sediment chemistry bulk and normalized values.

Statistical analysis allows the various test sediments to be ranked according to the differences in mortality and growth occurring among the sites. Spatial trends can be elucidated within a given study area and sites can be prioritized for remediation. Strategies for managing sediment can be based upon the severity of the biological effects detected in the laboratory and other supporting information outlined in the Provincial Sediment Quality Guidelines (Persaud *et al.*, 1992). To assist in the interpretation of the data, physical and physicochemical site-specific data, laboratory results and field studies are valuable in the overall sediment assessment.

The absolute tissue concentrations and sediment concentrations are reported. Tissue chemical concentrations can be compared between test and control animals but does not indicate whether the chemical has actually accumulated due to differences in sediment concentration, chemical desorption rates and organism uptake and elimination rate constants. Bioaccumulation factors (BAFs) are calculated for each chemical for the test and control sediments. The BAF is measured as the ratio of tissue concentration relative to the sediment concentration on a dry weight basis. Spearman rank correlation coefficients are derived for residue levels in biota and bulk sediment chemistry as well as corrected values based on percent fines and/or Total Organic Carbon for sediment and percent lipid for biota.

SECTION 6: LABORATORY SEDIMENT BIOASSAY METHODOLOGY

6.1 SCOPE

The procedures for performing laboratory sediment bioassays are described below. The procedures are intended to serve as a guide for conducting static, single-species, whole-sediment bioassays. The tests are designed to measure statistical differences among test and control sediments for the various biological endpoints.

The experimental unit is a 1.8 L test chamber containing one part of prepared sediment and four parts dechlorinated tap water, and is gently aerated (Figure 4). Each test chamber is an enclosed system with no exchange of test water with adjacent chambers. Chambers are randomly placed in a temperature controlled water-bath unit. The test organisms are randomly selected and introduced incrementally into the test chambers.

Test methodology has been previously described (Lomas and Krantzberg, 1988) and revised (Bedard, 1989; Krantzberg, 1990). Additional information was obtained from the literature (ASTM, 1990; Giesy *et al.*, 1988; Nebeker *et al.*, 1984; Mosher *et al.*, 1982).

The procedures for conducting both acute and chronic toxicity tests are described in Sections 6.2 to Section 6.11 with the following important exceptions:

Acute Lethality Test:

Section 6.6 - Test organisms are *H. limbata* and *P. promelas* only

Section 6.7 - Test duration is 10 days

Section 6.10 - Test endpoint is mortality. Follow Steps 1-3

Chronic Lethal and Sublethal Test:

Section 6.6 - Test organisms are *H. limbata*, *C. tentans* and *P. promelas*

Section 6.7 - Test duration is as follows:
H. limbata - 21 days
C. tentans - 10 days
P. promelas - 21 days

Section 6.10 - Test endpoints are mortality, growth and chemical bioaccumulation

Follow Steps 1 - 9

TEST CONDITIONS AND APPARATUS

Facilities Tests are carried out in a laboratory setting, in an area with controlled lighting and temperature, preferably ventilated.

Water Supply A consistent source of uncontaminated, high quality water is used in testing. Sources can include well water or dechlorinated municipal water. Water quality is analyzed semi-annually to ensure consistency (Appendix D).

Temperature Tests are to be carried out in a temperature controlled water-bath or an environmental chamber at $20^{\circ}C \pm 2^{\circ}C$.

Lighting Photoperiod is 16:8 hour, light:dark from fluorescent lights.

Cleaning Test chambers are machine-washed, acid rinsed, air-dried, rinsed with distilled water and a final rinse with test water.

Safety It is the responsibility of the user of this guide to consult the appropriate health and safety practices prior to use.

6.2 SEDIMENT COLLECTION AND STORAGE

1. The top 5 cm of bottom sediment are collected using a grab sampler such as a Ponar grab that will collect a relatively undisturbed sample from a range of sediment types.

2. Combine individual samples from a station until 10 L of sediment is obtained. Store sediment from each station in a 20 L bucket lined with a food-grade, polyethylene bag. Obtain larger volumes (15 L) for coarser sediments with a high gravel content or sediments that contain large amounts of debris e.g. twigs, fibrous material, clam shells.

3. Remove a subsample of sediment for chemical analysis and prepare according to procedures outlined in "A Guide to the Collection and Submission of Samples for Laboratory Analysis" (OMOE, 1989). See Section 6.4 for parameter list.

4. Tie the polyethylene bags using plastic ties in a manner that minimizes air space.

5. Seal the buckets and label with the station number, date, time and sampling depth.

6. Samples should be kept cool during shipping. Store sediments at 4°C in the dark prior to testing.
   Note: Biological testing should commence within 4 weeks after collection in order to minimize changes to sediment geochemistry (Othoudt *et al.*, 1991).

6.3 SEDIMENT PREPARATION

1. Decant any excess water from the sediment.

2. Press the wet sediment through a solvent-rinsed stainless steel sieve (US #10, 2 mm) to remove large debris and any large biota present. Collect sieved material into a clean, food-grade, polyethylene bag.

3. Homogenize the sieved sediment with a stainless steel spoon and transfer subsamples for future chemical and physical analysis according to procedures outlined in OMOE (1989).

4. Store remaining sediment at 4°C in 4 L glass jars with lids lined with hexane-rinsed foil.

6.4 SEDIMENT CHARACTERIZATION

Sediment characterization should include as a minimum the following analyses: loss on ignition, total organic carbon, percent moisture, particle size composition, total phosphorus, total Kjeldahl nitrogen, metals, PCBs and organochlorine pesticides. Additional parameters can be added on a site-specific basis and can include but are not restricted to chlorinated benzenes, PAHs, chlorinated dioxins and furans, chlorinated phenols and ammonia.

1. Submit samples of sediment for analysis that were prepared in Sections 6.2 and 6.3. The latter can be used to account for any changes that may have arisen due to storage and any differences in the collection of the subsamples for chemical analysis.

6.5 CONDUCTING THE TEST

1. Thoroughly homogenize previously prepared sediment in the 4 L glass jar using a stainless-steel spoon.

2. Measure a 325 mL aliquot of the homogenized sediment into a 400 mL glass beaker and transfer into a 1.8 L wide-mouth glass jar[1] (11.5 X 11.5 X 14.5 cm). Evenly distribute the sediment to a 2 cm depth by tapping the test chamber. Surface area of the sediment is 130 cm^2.

[1] 64 Fluid Oz Square® jars are available from Consolidated Bottle Co., Toronto, Ontario. 2 L glass beakers could serve as an appropriate substitute.

3. Wipe clean any excess material around the mouth of the test chamber and wash any sediment adhering to the inside of the jar with dechlorinated water.

4. Repeat steps 6.5.2 and 6.5.3 until each test sediment is allocated to a minimum of three, separate test chambers (for each species being tested).

5. Label each jar with the name of the study area, the appropriate station number and replicate.

6. Gently pour 1300 mL of dechlorinated tap water into each test chamber to achieve a 4:1 (v:v) water to sediment ratio.

7. Place the test chambers randomly into a temperature-controlled 20°C water-bath.
   Note: For sediments with high concentrations of contaminants or volatile substances, the tests should be carried out in a well-ventilated area or a fume hood at ambient room temperature.

8. Allow the sediment in the test chamber to settle and equilibrate overnight.

9. Place lids (10 cm; plastic or glass) over the test chambers to minimize evaporation and to the input of dust and debris.

10. Aerate the test chambers for a minimum of 1 hour prior to the introduction of the test organisms. Aeration rate is maintained by flow meters to create a steady stream of bubbles (1 meter regulating 6 test jars). Air is filtered to remove water and oil. Bubble air through Pasteur pipettes that extend 1 cm below the water surface and are retained with rubber o-rings through an opening in the plastic lid. Modifications may be necessary when using glass lids.

11. Maintain test chambers at 20°C, under a 16:8 hour, light:dark photoperiod and continuous aeration at a rate that ensures oxygen saturation.

12. All tests must include a negative control sediment and should include reference and positive controls.

6.6 HANDLING OF TEST ORGANISMS

Acute Lethality Test:

Test organisms are *H. limbata* and *P. promelas*.

Chronic Lethal and Sublethal Test:

Test organisms are *H. limbata*, *C. tentans* and *P. promelas*.

1. Unless state otherwise, all water used in this section is standard test water at room temperature.

2. Acclimation of test organisms is not required prior to transfer into the test chambers because the water supply, temperature and photoperiod are similar for culturing and test procedures.

3. Animals that are not healthy and active or injured or dropped during handling are rejected.

I. MAYFLY NYMPHS (HEXAGENIA LIMBATA)

The tests use 3 to 4 month old, reared mayfly nymphs with an average wet weight of 5 mg. Rearing methodology is described in Appendix A.

1. Place 2.4 L of dechlorinated water in an enamelled tray (20 X 40 cm) and aerate with a 2.5 cm air stone. Each tray can temporarily hold 200 nymphs.

2. Siphon off 80% of the overlying water from the 6.5 L rearing aquaria.

3. Remove 150 mL portions of bottom sediment and place the sediment into a 500 µm mesh brass sieve.

4. Separate nymphs from the sediment by immersing the sieve in a bucket of water and gently wash away the sediment by raising and lowering the sieve.

5. Place a 250 or 500 µm sieve into each enamelled tray. The screen will provide a surface to support the mayfly nymphs thus minimizing movement.

6. Wash the nymphs from the sieve into the prepared enamelled tray.

7. Sort and remove mayflies with the wide end (5 mm diameter) of a Pasteur pipette.

8. Transfer 5 nymphs into 100 mL glass beakers of water.

9. Gently pour the contents of the 100 mL beakers into the test chambers.

10. Repeat Steps 6.6.8 and 6.6.9 until each chamber contains 10 nymphs or a test density of 0.08 nymphs per cm^2.
    Note: Transfer should be completed within 1 to 2 hours after the mayflies were initially removed from the culture aquaria to minimize stress.

11. During sorting randomly select 30 to 50 individuals, weigh individually to the nearest 0.01 mg to obtain the initial average wet weight and then discard.

II. CHIRONOMID LARVAE (CHIRONOMUS TENTANS)

The tests use 10 to 12 day old, cultured chironomid larvae with an average wet weight of less than 1 mg. Culturing methodology is described in Appendix B.

1. Collect larvae from rearing pans by gently touching the sides of the larval tubes using a pair of feather-tipped forceps then removing with the wide end of a Pasteur pipette and release the animals below the water surface of the test chamber.

2. Transfer 7 or 8 larvae individually from the rearing pan into each of the test chambers.

3. Repeat Step 6.6.2 until each chamber contains 15 chironomids or a test density of 0.12 larvae per cm^2.

5. Examine the chambers within 18 hours and remove and replace any "floaters" (animals that get trapped in the surface film and die).
    Note: The turbulence generated from aeration may cause some floaters to settle to the bottom of the test chamber.

III. JUVENILE FATHEAD MINNOWS (PIMEPHALES PROMELAS)

The tests use cultured juvenile fathead minnows weighing 250-400 mg (wet wt.). Culturing methodology is described in Appendix C.

1. Count and sort 5 juvenile minnows into 250 mL glass beakers of water.

2. Empty the contents of the 250 mL beakers into a small minnow net and transfer minnows into the test chamber.

3. Repeat Steps 6.6.1 and 6.6.2 until each test chamber contains 10 minnows or a test density of 0.08 minnows per cm^2.

4. During sorting, a random subsample of 30 to 50 individuals are separated, weighed to the nearest 0.01 mg to obtain the initial average wet weight and then discard or submit for pre-exposure chemical analysis.

6.7 TEST DURATION

Acute Lethality Test:

Test Duration - 10 days - *H. limbata*

10 days - *P. promelas*

Chronic Lethal and Sublethal Test:

Test Duration - 21 days *H. limbata*
P. promelas
10 days *C. tentans*

1. The test begins when the animals are placed into the test chambers and is regarded as Day 0.

6.8 FEEDING REGIME

I. MAYFLY NYMPHS (HEXAGENIA LIMBATA)

1. Animals are not fed during the test.

II. CHIRONOMID LARVAE (CHIRONOMUS TENTANS)

1. Prepare a feeding solution by blending 0.9 g (dry wt.) Cerophyll®[2], 0.6 g (dry wt.) finely crushed Tetra Conditioning Food®[3] (3:2 w:w) and 100 mL dechlorinated water. Blend the mixture into a fine slurry .

2. Supply each test chamber a 2 mL aliquot (30 mg) of the vegetable diet solution on a daily basis.
   Note: If there is a build-up of food or fouling on the sediment surface, either temporarily suspend feeding or reduce to 1 mL.

[2] Cerophyll® cereal leaves is supplied by Ward's Natural Science, Mississauga, Ontario. Alfalfa powder can serve as a suitable substitute.
[3] Tetra Conditioning Food® is available at pet supply stores.

III. JUVENILE FATHEAD MINNOW (PIMEPHALES PROMELAS)

1. Each minnow receives a daily diet that is equivalent to 1% of the average wet weight of individuals in the subsample. See Step 6.6.5.

2. Prepare the feeding solution by mixing the calculated amount of coarsely crushed Tetra Conditioning Food® with dechlorinated water.

6.9 TEST MONITORING

1. Measure and record pH, conductivity and dissolved oxygen at the beginning, midway and end of the test. Additional water quality parameters may include hardness, alkalinity and ammonia. Measurements are made upon a different replicate for each interval. Record the temperature on a daily basis, preferably with a chart recorder.

2. Record the number of dead organisms on a daily basis. The dead organisms are removed and discarded.

3. Note any changes in the appearance of the test chambers during the test (e.g. turbidity).

4. Note any signs of stress or abnormal behaviour by the test organism e.g. burrowing activity.

5. Replenish the water loss due to evaporation with dechlorinated water as needed.

6.10 COMPLETION OF THE TEST AND RESPONSE CRITERIA

Acute Lethality Test:

Test Endpoint - Lethality
Follow Steps 1 - 3

Chronic Lethal and Sublethal Test:

Test Endpoints -
Lethality and Sublethal Growth Effects
Follow Steps 1 - 9

1. Empty the contents of the test chamber into a sieve bucket and gently rinse with dechlorinated water.

2. Wash the sieved material and organisms into an enamelled tray.

3. Sort and remove the surviving organisms with a pair of feather-tipped forceps. Count and record the number of surviving organisms.
   Note: Indicate the type and number of indigenous fauna that may be present.

4. Transfer all surviving test organisms from a single test chamber into a 150 mL beaker holding 100 mL dechlorinated water.

5. Immobilize mayfly nymphs or fathead minnows with CO_2 by adding 1/2 and 2 Alka-Seltzer® tablet(s), respectively, to the 150 mL beakers.

6. Chironomid larvae are placed into 150 mL beakers holding 100 mL dechlorinated water and 15 mL of silica sand.

7. Weigh individual mayfly nymphs and chironomid larvae to the nearest 0.01 mg. Record the fresh weight after removing the organisms from the beakers and briefly blotting dry on absorbent towels.
   Note: Alternatively, wet weight may be converted to dry weight using a correction factor or dry weights may be obtained after the animals have been dried to a constant dry weight. Length measurements can also be used as an indicator of chironomid and mayfly growth.

8. Pool the surviving fathead minnows into equal duplicate samples for each test sediment.

9. Transfer the minnows into 30 mL glass vials, label and keep frozen.

6.11 BIOTA ANALYSIS

1. Submit frozen fathead minnow samples for chemical analysis. The chemical concentrations are calculated as dry weight on unpurged animals. Lipid content should also be reported.

APPENDIX A: REARING PROCEDURES FOR THE MAYFLY, HEXAGENIA LIMBATA

SCOPE

The procedures that are undertaken at the OMOE laboratory for rearing the burrowing mayfly, *Hexagenia* are described. These procedures are intended to act as a general guide for rearing organisms under standard conditions and may require modifications to meet the specific needs of others.

Mayflies are reared from field-collected eggs which represent *H. limbata* and *H. rigida* species (J.J.H. Ciborowski, University of Windsor, personal communication). The ratio of the two species can be determined by the morphology of mature nymphs. The effect of using two species in toxicity tests is unknown but is expected to be minimal when considering the ecological and taxonomic similarities. Test organisms are randomly selected to represent a cross-section of the population.

The majority of the rearing procedures described are adaptations of Friesen (1981) and readers are referred to this document for more detail. Additional information was obtained from Fremling and Mauck (1980), Hanes *et al.*, (1990), Kovats and Ciborowski (1989), Nebeker *et al.* (1984) and work conducted in our laboratory.

REARING CONDITIONS

Facilities Cultures are maintained in a laboratory setting, in an area that has controlled lighting, stable temperature and free of excessive disturbances.

Water Supply Uncontaminated, high quality water as used in testing. Water quality is checked semi-annually to ensure consistency (Appendix D).

Temperature range 20 $\pm$ 2°C

Lighting photoperiod of 16:8 hour, light:dark from fluorescent lights.

Cleaning Culture apparatus are washed with a non-phosphate detergent solution, rinsed several times with tap water and allowed to air-dry.

1.0 OBTAINING TEST ORGANISMS

1.1 EQUIPMENT AND FACILITIES

1. Suitable water supply
2. Black light or other suitable light source
3. Potters' clay
4. Whirl-Pak® bags
5. Glass or plastic Petri dishes, 10 cm
6. Air supply, air line, 2.5 cm air stone
7. Low temperature incubator, 8°C

1.2 SOURCE

Mayfly eggs are obtained from Dr. J.J.H. Ciborowski at the University of Windsor. The eggs are collected on an annual basis in the months of June/July when the emergence of adult mayflies is at its' peak. The female imagoes are attracted by a black light at dusk along the southern shore of Lake St. Clair. The eggs are deposited when the female imagoes are placed upon water-filled containers. The eggs are then cooled to 8°C for storage following the procedures described below (see Section 1.3).

1.3 STORAGE OF EGGS

1. The freshly collected eggs are prepared for storage by transferring approximately 2,500 eggs into 250 mL Whirl-Pak® bags containing aerated, dechlorinated water.

2. Add a small amount of suspended clay into each bag. The clay helps minimize the clumping of eggs which can reduce hatching success.

3. Hold the eggs for 6.5 days at 20°C then reduce the temperature by 4°C every 4

days until 8°C is reached. An adjustable, low-temperature incubator is recommended.

4. The eggs can be stored at 8°C for up to 12 months. The eggs can be shipped in this state.

5. Check the temperature at which the eggs are being stored on a weekly basis.

6. Replace water loss due to evaporation with 8°C aerated, dechlorinated water.

2.0 INITIATION OF CULTURE

2.1 EQUIPMENT AND FACILITIES

1. Glass or plastic Petri dishes, 10 cm
2. Suitable water supply
3. Air supply, air line, 2.5 cm air stone
4. Autoclave
5. Dissecting microscope, (10 - 40 X)
6. Pasteur pipette with bulb
7. Glass beaker, 1 L
8. Field-collected sediment known to support mayflies
9. Enamelled trays, 20 X 40 cm
10. Fine-tipped forceps
11. Aquarium, 6.5 L
12. Plastic wrapping
13. Algal culture
14. Cerophyll®, (cereal leaves)
15. Tetra Conditioning Food®, (fish food flakes)

2.2 PREPARATION OF REARING FACILITIES

1. Place 900 mL of unsieved, refrigerated (4°C) sediment into a 1 L glass beaker. The rearing substrate is field-collected sediment from an area that is relatively clean and known to support mayfly populations. The sediment should be fine-textured (predominately silt/clay).

2. Each 1 L beaker holds enough substrate to rear 600 nymphs in a 6.5 L aquarium (30 X 12.5 X 17.5 cm) at a density of 1.6 nymphs per cm^2.

3. Autoclave the substrate at 107°C for 15 minutes.

4. Allow the substrate to cool and empty the contents into an enamelled tray(s) e.g. 1.8 L of sediment per 40 X 20 cm tray.

5. Air-dry the sediment for 24 hours; turn the material over with a stainless-steel spoon; dry for an additional 24 hours.

6. Place 900 mL of the air-dried, autoclaved sediment into a 6.5 L aquarium to create a 2 cm uniform depth.

7. Pour 5.6 L of ambient, dechlorinated tap water into the aquarium to provide a water depth of 15 cm. To help reduce sediment resuspension, gently pour the water over a Petri dish which is placed on the sediment surface.

8. Allow the sediment to settle and then aerate the aquarium with a 2.5 cm air stone suspended 2 cm off the bottom.

9. Aerate the aquarium for 6 to 7 days.

10. Gently scrape the sediment surface with a fork or a pair of tongs to aerate the lower layers of sediment with minimum sediment resuspension.

11. Aerate the aquarium an additional 3 to 5 days.

2.3 EMBRYO DEVELOPMENT

1. Aerate 500 mL of dechlorinated water for at least 24 hours.

2. Pour 15 mL aliquots of the aerated water into 10 cm Petri dishes.

3. Transfer approximately 300 eggs (Section 1.3) into each Petri dish using the wide end of a Pasteur pipette (5 mm diameter

opening). Prepare 3 Petri dishes for each culture aquarium.

4. Separate any clumped eggs using a pair of fine-tipped forceps with the aid of a dissecting microscope (40X). This will improve the hatching success.

5. Incubate the eggs at ambient room temperature for 7 to 14 days at which time hatching will begin. Hatching success is usually about 90%.

2.4 HANDLING OF ORGANISMS

1. Transfer newly hatched nymphs (< 24 h) from the Petri dishes (Section 1.3) and into the prepared rearing aquarium which has been aerated at least 10 days (Section 2.2.11).

2. Count the nymphs under a dissecting scope (10X) while drawing into a Pasteur pipette. For improved control during transfer, take the narrow end of the pipette and break it approximately half-way up.

3. Remove the air stone from the rearing aquarium during the transfer process.

4. Transfer 1 up to 25 nymphs at a time.

5. Gently release the nymphs 1 to 2 cm above the sediment surface with minimum disturbance of the animals and the sediment.

6. Replenish the hatching containers with aerated, dechlorinated water.

7. Continue transferring nymphs until each 6.5 L aquarium contains 600 nymphs at a density of 1.6 nymphs per cm^2. The eggs will continuously hatch for a period of 3 to 5 days. Estimated mortality during the first 3 months of development is 20-25%.

2.5 FEEDING AND MAINTENANCE

1. Maintain cultures at room temperature, 20 $\pm$ 3°C and a 16:8 hour, light:dark photoperiod and continuous aeration.

2. Initiate feeding on the fifth day following transfer (Section 2.3). Supply each 6.5 L aquarium with a 50 mL aliquot of an algal suspension, once or twice a week depending upon the age of the suspension. The suspension is comprised of *Selanastrum* (10%) and *Chlorella* (10%) species or an appropriate substitute may be used (Poirier *et al.*, 1988).

3. Supply each aquarium a 5 mL aliquot of a vegetable diet twice a week starting on the fifth week after transfer. Prepare the feeding solution by blending 3 g Cerophyll® powder (dry wt.) and 2 g finely crushed Tetra Conditioning Food® (dry wt.) in 80 mL of dechlorinated water. Remaining solution can be used for up to 7 days provided that it is refrigerated at 4°C.

4. Siphon 1 L or 25% of the culture water from each aquarium starting between Week 5 and 7, every three weeks. Replenish with fresh, ambient dechlorinated water being careful not to disturb the sediment surface.

5. The mayfly nymphs are available for testing purposes after 3 to 4 months (average wet wt., ~5 mg).

APPENDIX B: CULTURING PROCEDURES FOR THE MIDGE, CHIRONOMUS TENTANS

SCOPE

The procedures that are undertaken at the OMOE laboratory for culturing the midge larvae, *Chironomus tentans* are described. The procedures are intended to act as a general guide for culturing organisms under standard conditions and may require modifications to meet the needs of others.

Culture techniques are adapted from published literature which include Mosher *et al.* (1982), Nebeker *et al.* (1984), and Townsend *et al.* (1981) and work conducted in our laboratory.

CULTURING CONDITIONS

Facilities	Cultures are maintained in a laboratory setting, in an area that has controlled lighting, stable temperature and free of excessive disturbances.
Water Supply	Uncontaminated, high quality water as used in testing. Water quality is analyzed semi-annually to ensure consistency (Appendix D).
Temperature	range 20 $\pm$ 2°C
Lighting	photoperiod of 16:8 hour, light:dark from fluorescent lights.
Cleaning	Culture apparatus are washed with a non-phosphate detergent solution, rinsed several times with tap water and allowed to air-dry.

1.0 OBTAINING TEST ORGANISMS

1.1 EQUIPMENT AND FACILITIES

1. Suitable water supply
2. Artificial substrates, ie. weighboats, disposable pipettes
3. Aquarium lid, Plexiglass and mesh
4. Aspirator or suitable container
5. Aquarium, 6.5 L

1.2 SOURCE

Chironomid egg masses were obtained from stock cultures maintained by Dr. N. Collins and Dr. R. Baker at the University of Toronto. Additional egg cases were acquired from Dr. J. Giesy at Michigan State University. Other sources include federal agencies and local environmental consulting firms.

1.3 BREEDING AND EGG COLLECTION

1. Place 500 mL of dechlorinated water into a 6.5 L aquarium (30 X 12.5 X 17.5 cm) to a depth of 1 cm. This is to serve as a laying aquarium.
2. Add assorted artificial substrates such as 1 mL disposable pipettes and weighboats to the aquarium in order to create a favourable environment for egg deposition.
3. Tilt the aquarium on a slight angle (20°).
4. Cover the aquarium with a screened top to ensure the enclosure of the adult flies.
5. Transfer recently emerged flies from the culture aquarium (Section 2.5, Step 2.5.7) by using an aspirator or closed container with removable lid. Transfer adults into the laying aquarium.
6. Transfer adults in a 1:2 ratio (female:male). The male flies are distinguished by their feathery antennae and slender body. Males will usually emerge before the females.
7. Egg masses are deposited 1 to 2 days after the adults are transferred.
8. Transfer the egg masses into the prepared rearing pans (Section 2.2). Egg masses cannot be stored and remain viable for any extended period of time.

2.0 INITIATION OF CULTURE

2.1 EQUIPMENT AND FACILITIES

1. Enamelled tray, 20 X 40 cm
2. Suitable water supply
3. Air supply, air line, 2.5 cm air stone
4. Pasteur pipette with bulb
5. Plastic wrapping
6. Erlenmeyer flask, 100 mL or 250 mL
7. Adjustable or fixed volume pipet, 1 mL
8. Pipet tips, 1 mL
9. Tetra Conditioning Food® (fish food flakes)
10. Aquarium, 21 L
11. Silica sand, fine-grade
12. Feather-tipped forceps
13. Cerophyll® (cereal leaves)

2.2 EMBRYO DEVELOPMENT

1. Fill enamelled tray(s), with dechlorinated water to a depth of 2.5 cm.

2. Cover tray(s) with plastic wrapping which is supported to prevent the wrapping material adhering to the water surface.

3. Place a 2.5 cm air stone into each tray and aerate for 24 to 48 hours.

4. Permanently remove air stone from the tray at the time the egg masses are added.

5. Transfer 2 egg masses (approx. 1 cm in length) as described in Section 1.3 into each tray using the wide end of a Pasteur pipette (5mm diameter opening). Place egg masses that were deposited within 24 hours of each other into the same tray.

6. Monitor the egg masses for signs of development. The egg masses should begin to hatch 2 to 3 days after transfer. Each egg masses will produce at least 200 larvae.

7. Prepare the feeding solution by blending 2.5 mg of finely crushed Tetra Conditioning Food® in 80 mL of dechlorinated water.

8. When the egg masses begin to disintegrate and the larvae begin to disperse, feed daily as follows. Evenly disperse a 2 mL aliquot of feeding solution to each tray using a 1 mL disposable pipette.
   Note: Overfeeding should be avoided because Tetra Conditioning Food® has a tendency to foul the water. If this occurs then aeration or partial replacement of water will be required.

9. After 10 to 12 days larvae will be in the second instar (<1 mg, wet wt.) and are available for either testing or as a culture source.

2.3 PREPARATION OF CULTURING FACILITIES

1. Place 1.6 L of fine-grade, silica sand which has been previously rinsed with dechlorinated water, into a 21 L aquarium (40 X 20 X 25 cm).

2. Fill the aquarium with 8 L of ambient, dechlorinated water to a depth of 10 cm. Pour the water over a Petri dish placed on the substrate in order to reduce resuspension.

3. Aerate with a 2.5 cm air stone for 1 or 2 days.

2.4 HANDLING OF ORGANISMS

1. Transfer 250 second instar midge larvae (Section 2.2) preferably hatched from 2 or more egg masses, into the prepared 21 L aquarium (Section 2.3). The final density is 1 larvae per 3 cm^2. Individual larvae are retrieved from the rearing trays by gently touching the sides of the larval tubes with a pair of feather-tipped forceps, then

removing with the wide end of a Pasteur pipette (5mm diameter opening).

2. Label the culture aquarium with date and sample size.

2.5 FEEDING AND MAINTENANCE

1. Maintain cultures at room temperature, 20 ± 2°C and a 16:8 hour, light:dark photoperiod and continuous aeration.

2. Cover the aquarium with plastic wrapping.

3. Prepare the feeding solution by blending 3 g Cerophyll® powder (dry wt.) and 2 g finely crushed Tetra Conditioning Food® (dry wt.) in 80 mL of dechlorinated water. Blend the mixture into a fine slurry.

4. Initially provide newly transferred larvae with 10 to 20 mL of feeding solution, then 5 mL every other day. As the larvae grow, the quantity and/or feeding interval is adjusted to ensure an adequate food supply.

5. Siphon 1.5 L of the culture water or 20% of the total volume, every 2 weeks and replace with fresh, dechlorinated water.

6. After 2 weeks following transfer, remove the plastic wrap and cover the aquarium with a screened top constructed from Plexiglass and fine mesh. Place disposable pipettes in the culture aquarium above the water line to provide resting sites for emerging adults. The larvae will begin to pupate around 24 to 28 days after egg deposition and 2 days later adults will emerge. The screened top will contain the emerging adult flies.

7. Adults are then removed for egg deposition and for the initiation of new cultures (Section 1.3).

3.0 STORAGE OF LARVAE

3.1 EQUIPMENT AND FACILITIES

1. Incubator or adjustable water-bath
2. Cerophyll® (cereal leaves)
3. Tetra Conditioning Food® (fish food flakes)

3.2 FEEDING AND MAINTENANCE

Chironomid cultures must be continuously maintained through the rearing and initiation of new cultures due to the inability to store the egg masses on a long-term basis. Therefore, it may be necessary to arrest the development of the midge larvae for short periods of time.

1. Gradually subject a standard culture of second and third instar (2 - 3 week old) larvae (Section 2.3 to 2.5), to cooler temperatures by reducing the temperature from 20°C to 8°C in 2°C increments every day. A temperature controlled water-bath or an adjustable incubator could be used.

2. The culture is stored in complete darkness at 8°C and is not aerated.

3. Feed the larvae 5 - 10 mL amounts of a Cerophyll®:Tetra Conditioning Food® mixture (3:2, w:w), e.g. 3 g Cerophyll and 2 g Tetra Conditioning Food in 80 mL of dechlorinated. The larvae are fed *ad libitum*.

4. When required, growth and development can be induced by gradually acclimating the larvae to room temperature in 2°C increments each day.

APPENDIX C: CULTURING PROCEDURES FOR THE FATHEAD MINNOW, PIMEPHALES PROMELAS

SCOPE

The procedures that are undertaken at the OMOE laboratory for culturing the fathead minnow, *Pimephales promelas* are described. The procedures are intended to act as a general guide for culturing organisms under standard conditions and may require modifications to meet the specific needs of others.

Culture techniques for the most part follow the USEPA protocol (USEPA, 1987) with some minor deviations and the following is an overview. Readers are referred to above document for more detail.

CULTURING CONDITIONS

Facilities	Cultures are maintained in a laboratory setting, in an area that has controlled lighting, stable temperature and free of excessive disturbances.
Water Supply	Uncontaminated, high quality water as used in testing. Water quality is analyzed semi-annually to ensure consistency (Appendix D).
Temperature	range 25 $\pm$ 2°C
Lighting	photoperiod of 16:8 hour, light:dark from fluorescent lights.
Cleaning	Culture apparatus are washed with a non-phosphate detergent solution, rinsed several times with tap water and allowed to air-dry.

1.0 OBTAINING TEST ORGANISMS

1.1 EQUIPMENT AND FACILITIES

1. Suitable water supply, 25°C, flow-through
2. Aquarium, 60 L
3. Air supply, air line, 2.5 cm air stone
4. Plastic screen
5. PVC pipe, 11 cm O.D.

1.2 SOURCE

The OMOE, Toxicity Unit cultures juvenile fathead minnows for toxicity testing at the Rexdale laboratory facility under the supervision of Dr. G. Westlake and direction of D. Poirier.

The original source of breeding stock was the USEPA laboratory in Duluth. Periodically, additional wild stock have been acquired from ponds in central Ontario. These animals are quarantined for one year prior to being introduced into the breeding cycle.

1.3 BREEDING AND EGG COLLECTION

1. Set aside a group of juvenile minnows to serve as breeding stock (Section 2.0) and hold in 60 L glass aquaria (31 X 61 X 32 cm).

2. Maintain the aquaria at 25°C in a flow-through (5 L h^{-1}), dechlorinated water system and under a 16:8 hour, light:dark photoperiod.

3. Transfer paired mature males and females into a spawning chamber. A 60 L glass aquarium is divided into 2 spawning chambers separated by a plastic screen. Mature fish are approximately 4 to 8 months of age.
   Note: The OMOE laboratory maintains 30 pairs of breeders.

4. Occasionally the brood stock is induced to spawn by lowering the water temperature by 2°C per day to 10°C for 7 days and reducing the photoperiod to 8:16 hour, light:dark. The temperature and light are returned to 25°C and 16:8 hour, light:dark over 7 days for spawning.

5. Construct the spawning tiles by cutting a 11 cm (O.D.) PVC pipe into 7.5 cm lengths. The lengths are halved to form an inverted tunnel. The inside surface is roughen with sandpaper. Alternatively, use clay pipe cut similarly.

6. Place 1 spawning tile into each spawning chamber.

7. Check the underside of the spawning tile on a daily basis for egg deposition.

8. Each spawning pair will typically produce 75-200 eggs a week for about 2 months, at which time the mating pair is replaced with fresh spawners.

2.0 INITIATION OF CULTURE

2.1 EQUIPMENT AND FACILITIES

1. Aquarium, 60 L
2. Fibreglass tank, 400 L
3. Suitable water supply, 25°C, flow-through
4. Air supply, air stone, air line
5. Water-bath, temperature controlled, 25°C
6. Glass beaker, 400 mL
7. Separatory funnel, 1 L
8. *Artemia* sp. eggs
9. Frozen brine shrimp
10. Sodium chloride
11. Disposable pipette, 1 mL

2.2 EMBRYO HATCHING AND DEVELOPMENT

1. Check the spawning tiles daily for the presence of embryos.

2. Transfer each tile containing embryos into a 400 mL beaker (Section 2.3).

3. Incubate the spawning tiles in the 25°C water bath and maintain aeration.

4. Replace those spawning tiles that have been removed from the spawning chambers with clean tiles for further egg deposition.

5. Check the embryos for signs of development and fungal growth. Remove fungoused eggs on a daily basis.

6. The embryos will begin to develop after 2 days and hatch within 5 days.

7. Transfer larvae into 60 L aquaria with a flow-through water supply.

8. After 1 - 1.5 months of development, juveniles are transferred into 400 L fibreglass holding tanks with a flow-through water supply.

2.3 FEEDING AND MAINTENANCE

1. Maintain larvae (<1-1.5 months) and juveniles (>1-1.5 months) at 25°C in a flow-through, dechlorinated water system and at a 16:8 hour, light:dark photoperiod and continuous aeration.

2. Prepare the larval feeding solution by placing 3 g *Artemia* sp. eggs, 1 L dechlorinated water and 5 g salt into a 1 L separatory funnel.

3. Vigorously aerate the solution for 48 hours at room temperature (20-23°C) through a 1 mL disposable pipette.

4. After 48 hours, allow the solution to settle and siphon off the settled organisms.

5. Feed the larvae (Section 2.3.1) live brine shrimp (48 hour) *ad libitum* once per day.

6. Prepare the feeding solution for the juveniles and breeders by placing appropriate quantities of frozen brine shrimp into dechlorinated water and allow the brine shrimp to thaw.

7. The brine shrimp feeding solution is supplied *ad libitum* once per day to the breeders (Section 1.3), juvenile fish (>1-1.5 months) and to the larval fish one week prior to their transfer (Section 2.3).

APPENDIX D WATER QUALITY PARAMETERS

Characteristics of dechlorinated Toronto tap water.

	mg L^{-1}
Calcium	42.0
Magnesium	9.0
Sodium	14.0
Potassium	1.5
Copper	0.2
Nickel	0.2
Lead	< T
Zinc	< T
Iron	< W
Chloride	29.0
Sulphate	30.0
Fluoride	1.1
Total Kjeldahl Nitrogen	< T
Total Ammonium	< W
Total Nitrate	0.5
Total Hardness, as $CaCo_3$	144
Dissolved Organic Carbon	1.1
pH	8.2
Conductivity, umho cm^{-1}	357

<T A measurable trace amount
<W No measurable response

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